



# Survival of *Mycobacterium avium* subsp. *paratuberculosis* in biofilms on livestock watering trough materials

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## ABSTRACT

*Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is the causative agent of Johne's disease, a chronic enteric infection that affects ruminants. Despite the ubiquitous occurrence of *Mycobacterium* sp. in nature and the fact that Johne's disease has been reported worldwide, little research has been done to assess its survival in agricultural environments. The goal of this 365-day study was to evaluate the ability of *Map* to persist in mixed-community biofilms on materials commonly used to construct livestock watering troughs. *Map* was inoculated into 32 l of trough water containing either concrete, plastic, galvanized or stainless steel trough materials. The concentration of *Map* was determined by using quantitative, real-time PCR to target the IS900 sequence in DNA extracts. High concentrations of *Map* were detected on all trough materials after 3 days (around  $1 \times 10^5$  cells  $\text{cm}^{-2}$ ). Based on the best-fit slopes, the time required for a 99% reduction ( $t_{99}$ ) in biofilm-associated *Map* cells was 144 and 115 days for plastic and stainless steel trough materials, respectively. *Map* concentrations did not decrease on concrete and galvanized steel trough materials. These results suggest that *Map* survives well in biofilms present on livestock watering trough materials. To inhibit spread of this organism and exposure of susceptible animals to *Map* on infected farms, best management practices aimed at maintaining biofilm-free trough surfaces should be included in any Johne's control plan.

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## 1. Introduction

*Mycobacterium avium* subsp. *paratuberculosis* (*Map*), the causative agent of Johne's disease, is considered one of the most serious diseases of dairy cattle. Johne's disease has been reported worldwide and has caused devastating economic losses resulting from premature culling and reduced milk production from infected animals (Harris and Barletta, 2001; Rowe and Grant, 2006). *Map* is transmitted through a fecal–oral route, including indirect transmission via ingestion of contaminated materials in the farm environment (Lombard et al., 2006). Since infected cattle

can release as many as  $10^8$  cells/g of feces, the presence of shedding animals on the farm increases the likelihood of environmental exposure of other livestock (Johnson-Ifeorlundu and Kaneene, 1997; Whittington et al., 2004).

Although it is considered an obligate pathogen, *Map* survives well in the environment (200–600 days) possibly by entering a dormant state or by living in close association with other organisms (Cook and Britt, 2007; Pickup et al., 2005; Whittington et al., 2005). *Map* has been found in many locations in the agricultural environment (Berghaus et al., 2006; Johnson-Ifeorlundu and Kaneene, 1997; Pickup et al., 2005; Raizman et al., 2004; Whittington et al., 2005). In fact, recent studies suggest that environmental sampling for *Map* may serve as a viable alternative to individual animal testing since it accurately predicts herd status and is lower cost and easier to perform than herd

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testing (Berghaus et al., 2006; Lombard et al., 2006; Raizman et al., 2004; NAHMS, 2008).

Survival of *Map* in the farm environment may be aided by its ability to become established and persist in biofilms on moist surfaces until ingestion by a suitable host (Rowe and Grant, 2006). Studies have shown that mycobacteria are capable of rapid and sustained biofilm formation (Dailloux et al., 2003; Hall-Stoodley and Lappin-Scott, 1998; Steed and Falkinham, 2006; Torvinen et al., 2007). The extremely hydrophobic cell wall structure of these organisms increases their ability to adhere to surfaces (Steed and Falkinham, 2006). Research conducted by Bolster et al. (2008) showed that the cell wall of *Map* is also extremely hydrophobic, suggesting that the organism may be predisposed to colonization of surfaces. Therefore, the goal of this study was to evaluate the ability of *Map* to become established and persist in multi-species biofilms on plastic, concrete, galvanized and stainless steel livestock drinking water trough materials.

## 2. Materials and methods

### 2.1. Bacterial culture preparation

A culture of *Map* isolated from the ileum of a clinically infected, Johne's positive dairy cow was inoculated into flasks containing Middlebrooks 7H9 broth (Remel, Lenexa, KS; 1 l total volume) with 2 ml l<sup>-1</sup> glycerol, 10% Middlebrooks OADC (Becton Dickinson, Sparks, MD) and 2 mg l<sup>-1</sup> Mycobactin J (Allied Monitor, Fayette, MO). Cells were grown for 21 days at 37 °C with constant mixing. The culture was pelleted and washed three times in sterile phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.4 mM KH<sub>2</sub>PO<sub>4</sub>) by centrifugation at 4251 × g for 30 min. Cell clumps were disrupted by sonicating (Model 100 Sonic Dismembrator, Fisher Scientific, St. Louis, MO) three times for 15 s on a setting of 1 with 5 min between each successive sonication. The final pellet was re-suspended in 100 ml PBS. Twelve ml of this inoculum ( $3.3 \pm 0.47 \times 10^7$  cells ml<sup>-1</sup>) was used to inoculate each trough on day 0 of the experiment.

### 2.2. Trough setup, trough material preparation and trough water addition

Glass tanks (36 l; All Glass Manufacturing, Franklin, WI) were filled with 32 l of trough water (diluted in half with tap water) which was collected from a dairy farm located in western Kentucky. The trough water for the farm is supplied from city drinking water and the troughs on the farm are of the plastic variety. Glass tanks were used to hold the trough water and the different watering trough materials in a common, inert vessel. Trough materials were not mixed in the tanks, to avoid chemical interactions and were designed to represent the most commonly used livestock watering trough materials; plastic (Rubbermaid, Fairlawn, OH), concrete (Quikrete concrete, Atlanta, GA), stainless (McMaster-Carr, 0.064 cm thick, 1.27 cm wide, 25.4 cm coil, Aurora, OH) and galvanized (McMaster-Carr, low carbon sheet 0.061 cm thick, 121.9 cm wide, 121.9 cm long) steel trough materials. To supply surface area for

biofilm formation and provide uniform, independent samples, the trough materials were cut into 3.8 cm × 1.5 cm plates. Concrete coupons were produced using wooden molds of the same dimensions. Three trough material plates of the same type were strung vertically using Shakespeare® monofilament fishing line (6.8 kg test line; Columbia, SC) and were then tied to 1.27 cm wooden dowel rods and placed into the glass tanks (over 100 per tank). The tanks containing the trough material plates were maintained in the dark in large incubators (Perceval Scientific, Inc., Perry, IA) at 25 °C. Moisture was not controlled, but pans filled with deionized water were kept in the chambers near the fans to humidify the chambers and reduce trough water evaporation. Fresh trough water (undiluted) was added as needed to maintain tank volume at or around 32 l. Every time trough water was taken from the farm and added to the trough water tanks, 20 ml was extracted and analyzed for *Map* as described below. *Map* was never detected in trough water collected from the farm and trough water addition had no effect on total or *Map* cell concentrations in the biofilms or in the tank trough water (data not shown). Trough water pH was determined using a combination electrode (Fisher Scientific, Hampton, NH). Trough water pH was measured in the tanks prior to most samplings.

### 2.3. Sample collection and DNA extraction

*Map* was added to the trough water tanks on day 0. The water in the tanks (20 ml) was sampled on day 0 to determine the initial concentration of *Map* in the water. Thereafter, water samples (20 or 30 ml) were taken at the same time as trough material samples. Triplicate, randomly selected plates of each of the different trough materials were collected on day 3 and then weekly for 6 weeks. Thereafter, samples were taken on weeks: 10, 14, 21, 27, 34, 40 and 52. Trough material samples were placed in 50 ml centrifuge tubes (Falcon, Franklin Lakes, NJ) containing 10 ml of filter-sterilized deionized water with approximately 10–20 glass beads (Fisher Scientific, Pittsburgh, PA) and vortexed on high speed for 2 min. Supernatant was removed and filtered onto 0.2 µm white polycarbonate filters (Millipore, Billerica, MA). Filters were placed into lysing matrix with sodium phosphate buffer and DNA was extracted with the Qbiogene FastDNA® Spin Kit for soil (Qbiogene, Irvine, CA) according to manufacturer's specifications.

### 2.4. Re-inoculation of tanks with *Map*

Upon completion of sampling for the previous study, 12 ml of a fresh culture of *Map* ( $1.7 \pm 0.34 \times 10^9$  cells ml<sup>-1</sup>) was added to the trough water containing the remaining trough materials with biofilms established over the previous 365 days. These troughs had originally been inoculated with *Map* 365 days prior to this re-inoculation. Trough materials from the re-inoculated systems were sampled after 33 days and processed as described above for molecular analysis of total and *Map* cell numbers. *Mycobacteria* were visualized in the mixed-community biofilms on trough materials stained using the Fluka Fluorescent Stain Kit for *Mycobacteria*

(Sigma–Aldrich, St. Louis, MO) as described by manufacturer. Stained trough materials were viewed using an Olympus BX-41 microscope (Melville, NY) equipped with a 100-W mercury lamp and Olympus U-MNG (ex: 530–550 nm) and Olympus U-MWB (ex: 450–480 nm) filters. Auramine O-stained *Mycobacteria* fluoresced yellow and counterstained biomass is shown in orange. Images were obtained using a PowerShot S2 IS Canon camera (Lake Success, NY) and ProlImage (Silver Spring, MD) image analysis software.

## 2.5. Quantification of total and *Map* cells

Quantitative, real-time PCR (qPCR) was used to determine the concentration of total bacteria (16S rRNA gene) and *Map* (IS900 sequence). Assays were run on the DNA Engine Opticon 2 (MJ Research, Inc., Waltham, MA). The primers were obtained from Sigma Genosys (St. Louis, MO) and the dual-labeled Black Hole Quencher probes were prepared by Biosearch Technologies, Inc. (Novato, CA). Assays were carried out in Qiagen HotStart Taq Master Mix (Qiagen, Valencia, CA) in a total volume of 25  $\mu$ l. Template DNA consisted of DNA extract (4–20 ng) run in triplicate, with all PCR runs including duplicates of standards and control reactions without template. DNA concentrations were measured with the Hoechst 33258 nucleic acid stain (Invitrogen, Carlsbad, CA) and measured with a Hoefer DyNA Quant 200 fluorometer (Amersham Biosciences, San Francisco, CA) according to manufacturers instructions. *Map* concentrations were measured using qPCR targeting a 76 bp fragment of the *Map*-specific IS900 sequence. The assay was a modification of that developed by Kim et al. (2002), as previously described (Cook and Britt, 2007). Standard DNA consisted of plasmid PCR 2.1 vector (Invitrogen, Carlsbad, CA) carrying an IS900 insert from a larger fragment of the *Map* IS900 sequence (354 bp) amplified using primers F1 and R1 and PCR conditions as described by O'Mahony and Hill (2004). Cell concentrations were calculated by dividing the copy number per  $\text{cm}^2$  of trough material by the number of IS900 copies per cell (14–20; 14 was used in this study).

qPCR analysis of total cells (targeting 16S rRNA copies) was carried out as previously described (Harms et al., 2003) using the Qiagen HotStarTaq<sup>®</sup> Master Mix (Qiagen, Valencia, CA). The amplification mixture contained 3.0 mM  $\text{MgCl}_2$ , 600 nM each primer, 200 nM of probe and sample DNA or standard consisting of plasmid vector containing a 16S rRNA gene insert (1.46 kb;  $10^2$ – $10^8$  copies). The QRT-PCR program was 15 min at 95 °C, 39 cycles at 95 °C for 15 s, 58 °C for 45 s and 72 °C for 45 s. Cell concentrations were calculated by dividing the copy number per  $\text{cm}^2$  of trough material by 4.0, the average copy number of 16S rRNA genes per cell (Klappenbach et al., 2001).

## 2.6. Statistical analysis

Differences between treatments were analyzed by comparing fits to the log-linear portion of the cell concentration data (occurring from day 3 to day 149) obtained with a first-order decay model:

$$C_t = C_0 e^{-kt} \quad (1)$$

where  $C_t$  is the measured cell concentration at time  $t$ ,  $C_0$  is the cell concentration at time zero, and  $k$  is the decay rate; all analyses were performed by fitting a linearized version of Eq. (1) to log-transformed concentration data. Analysis of covariance (ANCOVA) was used to obtain parameter estimates and uncertainties for both  $k$  and  $C_0$  and to statistically compare the fitted values of the decay rates using an  $F$ -test for homogeneity. Error for these tests was based on two components: replicate samples for each treatment mean and lack of fit between the observed treatment means and predicted cell concentrations over sampling day. PROC MIXED in SAS was used to perform the ANCOVA and provide estimates for slopes and both sources of error (SAS Version 9.1; SAS Institute, 2003).

## 3. Results

### 3.1. Biofilm formation on trough materials

Trough water from a working farm was used as the source of liquid in all tanks and was used to replenish any liquid lost during the course of the study. By using trough water from the farm, the tanks were inoculated with the natural microbial flora present in the troughs. *Map* was never detected in the trough water from the farm. *Map* concentrations on day 0 in the inoculated trough water were  $4.6 \pm 2.8 \times 10^4$  cells  $\text{ml}^{-1}$ . For the first 14 days of the experiment the concentration of *Map* in the liquid suspension remained between 4 and  $6 \times 10^4$  cells  $\text{ml}^{-1}$ . Thereafter, concentrations in suspension were generally less than 1% of the original (at or near detection limits; data not shown).

Within 3 days of trough water addition, total cell concentrations on the trough materials averaged  $4.3 \pm 2.0 \times 10^5$  cells  $\text{cm}^{-2}$ . Biofilm total cell concentrations over the course of the 365 day study were highest on concrete and galvanized steel trough materials (averaging  $1.4 \pm 0.77 \times 10^6$  and  $2.4 \pm 0.71 \times 10^6$  cells  $\text{cm}^{-2}$ , respectively), while biofilm concentrations on plastic and stainless steel trough materials were lower (averaging  $5.4 \pm 2.6 \times 10^5$  and  $1.5 \pm 1.7 \times 10^5$  cells  $\text{cm}^{-2}$ , respectively) (Fig. 1). Trough water pH values averaged 7.67  $\pm$  0.46, 7.71  $\pm$  0.33, 7.82  $\pm$  0.35, 8.08  $\pm$  0.37 for plastic, galvanized steel, stainless steel and concrete coupons, respectively. pH was consistently higher for water in the systems containing concrete trough materials.

### 3.2. *Map* biofilm formation on trough materials

*Map* was added on Day 0 at a final concentration of approximately  $4.6 \pm 2.8 \times 10^4$  cells  $\text{ml}^{-1}$  and within 3 days, the concentration of *Map* in biofilms on the trough materials was  $9.0 \pm 5.1 \times 10^4$  cells  $\text{cm}^{-2}$ . *Map* concentrations generally stayed well above the detection limits (around  $5 \times 10^1$  copies; based on non-specific background levels in trough water control samples). However, the concentration of *Map* varied depending on the trough material used (Fig. 1). Only stainless steel materials contained *Map* concentrations that were at or near detection limits at any time (Fig. 1). *Map* was present on all trough materials sampled throughout the 365-day study.

*Map* concentrations ranged from 7% to 50% (averaging around 20%) of the total biofilm population. After 21 days, *Map* was less than 10% of the total biofilm community on

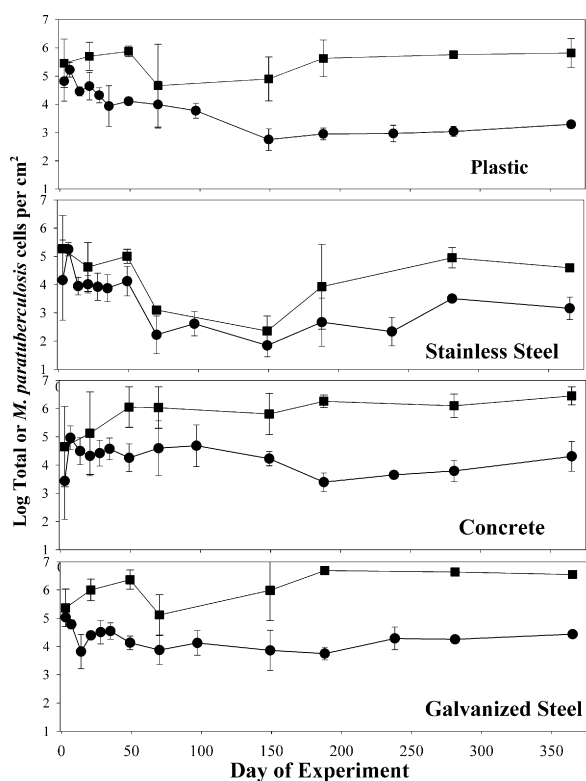


Fig. 1. qPCR analysis of *Map* (●) or total (■) cells on plastic, stainless steel, concrete or galvanized steel trough materials. Values are the mean  $\pm$  standard deviation of triplicate samples, each run in duplicate.

all materials except stainless steel. *Map* made up between 10% and 30% of the total biofilm population on stainless steel trough materials for the first 150 days before decreasing to 2% or less of the population.

### 3.3. Decay rates for *Map* on trough materials

Decay rates of *Map* on trough materials were calculated by fitting a linearized version of Eq. (1) to log-transformed concentration data. The largest decay rates were measured for the stainless steel and plastic trough materials whereas the lowest decay rates were measured with the galvanized steel and concrete materials (Table 1). Based on the ANCOVA, decay rates for *Map* on both the plastic and stainless steel coupons were significantly greater than zero ( $P < 0.01$ ) and were both significantly greater than the decay rates measured on the concrete and galvanized steel materials ( $P < 0.01$ ) but were not significantly ( $P > 0.05$ ) different from each other. On the other hand, the decay rates obtained for the concrete and galvanized steel coupons were not significantly different from zero ( $P > 0.05$ ), though the fitted decay rate for the galvanized coupon was not far from being statistically significant ( $P = 0.057$ ).

Based on the best-fit slopes, the time required for a 99% reduction ( $t_{99}$ ) in biofilm-associated *Map* cells on the plastic and stainless steel trough materials was 144 and 115 days, respectively.  $t_{99}$  values were not calculated for the concrete and galvanized steel trough materials because

Table 1

Decay rate (slope) and time required for a 99% reduction ( $t_{99}$ ) in concentration of *Map* on trough materials.

Trough material	$K^a$	$t_{99}$ (days) <sup>b</sup>	$P^c$
Concrete	$0.008 \pm 0.0051^{\S}$	N.C.	0.150
Galvanized steel	$0.012 \pm 0.0054^{\S}$	N.C.	0.057
Plastic	$0.032 \pm 0.0068^a$	144	0.001
Stainless steel	$0.040 \pm 0.0067^a$	115	<0.001

<sup>a</sup> Obtained by fitting a linear model to log-transformed cell concentration data ( $\pm$  standard errors). Values with the same symbol ( $\square$  or  $\S$ ) are not significantly different at the  $P = 0.05$  level.

<sup>b</sup> The time required for a 99% reduction in biofilm-associated cells ( $t_{99}$ ). Because die-off rates for concrete and galvanized steel were not significantly different,  $t_{99}$  were not calculated (N.C.).

<sup>c</sup>  $P$  = probability that the value of the fitted slope is significantly different from zero.

the fitted decay rates for these materials were not significantly greater than zero; findings consistent with our observation that overall concentrations of *Map* on concrete and galvanized steel materials did not decrease noticeably during the duration of the study (Fig. 1).

### 3.4. Re-inoculation of *Map* into troughs

After the day 365 sampling, troughs were re-inoculated with *Map* at a final concentration of approximately  $6.0 \times 10^5$  cells ml<sup>-1</sup>. Trough materials were sampled 33 days later. Fluorescent acid-fast staining specific for mycobacteria was used to visualize adherent cells on galvanized and stainless steel materials. Although this stain is not specific for *Map*, the images show that mycobacteria were intimately associated with the biofilms on these trough materials (Fig. 2). qPCR was used to quantify *Map* concentrations on the re-inoculated trough materials. Results show that concentrations increased on all trough materials except concrete (Table 2). These results suggest that the organism is able to become incorporated into established trough biofilms.

## 4. Discussion

The occurrence of *Map* in trough water has, to our knowledge, never been evaluated (Rowe and Grant, 2006). This despite several recent studies which suggest that environmental occurrence of the organism is highly correlated with the incidence of Johne's disease in livestock herds (Berghaus et al., 2006; Lombard et al., 2006; Raizman et al., 2004; NAHMS, 2008). Two important aspects of drinking water microbiology emphasize the additional need for studies of the survival of *Map* in livestock drinking water systems: (1) the well-documented ability of pathogens such as *Escherichia coli* O157:H7 to survive in and be transmitted to other animals from drinking water troughs and (2) the equally well-documented efficiency with which mycobacteria form biofilms on metal and plastic surfaces. The goal of this study was to evaluate the ability of *Map* to form mixed-community biofilms on materials commonly used to construct livestock watering troughs, to persist amid the trough water microbial flora and to



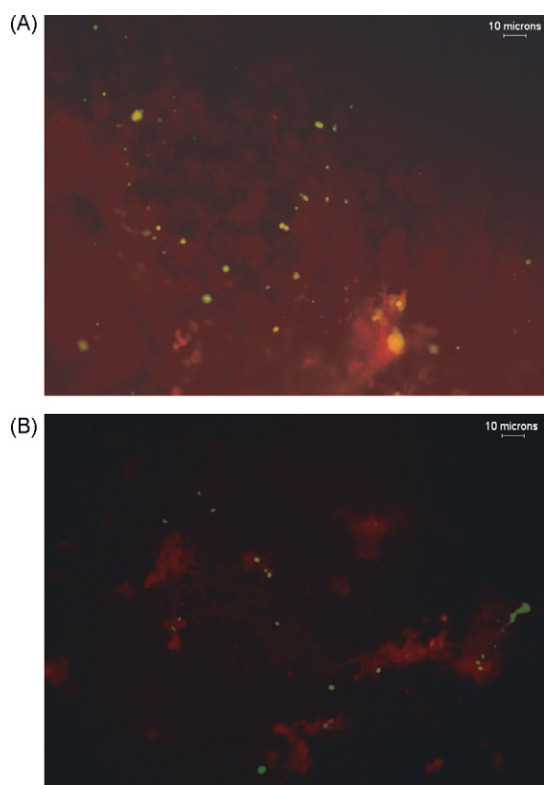


Fig. 2. Epifluorescence photomicrograph of Auramine O-stained mycobacterial cells (yellow) in a background of counterstained biomass (orange). The cells were present on galvanized (A) or stainless (B) steel trough materials 33 days after *Map* was re-inoculated into mixed-community biofilms. The stain was not specific for *Map* and therefore shows any potential environmental mycobacteria as well as *Map*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

become incorporated into an established biofilm on the same trough materials.

Drinking water troughs have been shown to harbor pathogens such as *E. coli* O157:H7 (McGee et al., 2002; Sargeant et al., 2003; Shere et al., 1998). In a study of 73 feedlots and over 3000 water or water-tank sediment samples, Sargeant et al. (2003) found that 13.1% of the water tanks were positive for *E. coli* O157:H7, and 60.3% of feedlots had at least one positive tank. Furthermore, cattle in pens with positive water tanks were more likely to be

Table 2

Cell numbers present on trough materials 33 days after re-inoculation with *Map*.

Material	Total (cells cm <sup>-2</sup> )	<i>Map</i> <sup>a</sup> (cells cm <sup>-2</sup> )	Ratio <sup>b</sup> (%)	Increase <sup>c</sup> (%)
Plastic	1.6 ± 0.7 × 10 <sup>6</sup>	1.0 ± 0.4 × 10 <sup>4</sup>	0.70	81
Stainless	1.3 ± 1.3 × 10 <sup>5</sup>	4.8 ± 4.3 × 10 <sup>3</sup>	3.60	60
Concrete	5.5 ± 8.9 × 10 <sup>5</sup>	2.7 ± 4.2 × 10 <sup>4</sup>	4.90	−40
Galvanized	8.8 ± 7.7 × 10 <sup>6</sup>	1.4 ± 1.0 × 10 <sup>5</sup>	1.50	79

Average ± standard deviation of cell numbers.

<sup>a</sup> *Map* cell numbers.

<sup>b</sup> Ratio = average portion of total coupon population that is *Map*.

<sup>c</sup> Increase in *Map* concentrations 33 days after re-inoculation.

shedding *E. coli* O157:H7. None of these studies have evaluated biofilm formation on livestock watering trough materials or the occurrence of *Map* in trough water. However, Lejeune et al. (2001) did include trough construction material as part of a survey of drinking water quality in 467 troughs. They found the distribution to be: 139 metal, 232 concrete, 88 plastic and eight other materials. The use of galvanized and stainless steel, concrete and plastic materials in this study was meant to reflect the major trough materials in use today.

Biofilms containing  $2 \times 10^5$ – $6 \times 10^5$  cells cm<sup>-2</sup> were detected within 3 days of addition of trough water. Concrete and galvanized steel trough materials contained the most concentrated biofilms, while concentrations on stainless steel and plastic were often more than 90% lower (Fig. 1). Previous research has shown that biofilm formation depends on complex interactions between the organism, the surface being colonized and the environment (Flemming, 2002). Lejeune et al. (2001) found that coliform and *E. coli* counts were significantly lower for metal troughs than for other materials (including plastic). It is well known that rough surfaces are more prone to colonization than are smooth surfaces (Faille et al., 2002; Flemming, 2002). In this study, adhesion of organisms to trough materials varied significantly and may have been influenced by surface properties of the construction materials.

*Map* was detected in biofilms for 365 days. *Map* did not disappear from the biofilms in a linear fashion, but concentrations reached a plateau at or near day 149 (Fig. 1). In this study, the mechanisms by which *Map* was able to become established and persist in biofilms on the trough materials were not studied. However, previous studies have shown that the hydrophobic nature of the mycobacterial cell wall may predispose these organisms to adherence (Bolster et al., 2008; Dailloux et al., 2003; Tatchou-Nyamsi-Konig et al., 2008; VanLoosdrecht et al., 1987). Bolster et al. (2008), investigating processes controlling the transport of *Map* through aquifer materials, confirmed that the cell wall of *Map*, like other mycobacteria, has a strong negative charge and is highly hydrophobic. They found that *Map* was poorly transported through saturated porous material and suggested that the hydrophobicity of the cell wall may contribute to its adhesion. In a study of bacterial adhesion to inert surfaces, Faille et al. (2002) found that the strength of adhesion depended on the surface properties of the organism and the material being colonized (i.e., surface roughness and free energy). Lower adhesion of *Map* to stainless steel and plastic surfaces may be due to weaker interactions between the hydrophobic cell wall of *Map* and the inert surfaces of those materials.

In addition to adhesion, *Map* may also be maintained through associations with other organisms within the biofilm. Studies have shown that the *Map* may be capable of surviving for weeks within environmental protozoa and that this may serve as an environmental survival mechanism for the organism (Mura et al., 2006; Rowe and Grant, 2006; Whan et al., 2006). Given results from this study, further research to evaluate mechanisms of survival and infectivity of *M. paratuberculosis*

following incubation in trough water biofilms is warranted.

*Map* rapidly (within 3 days) incorporated into biofilms on all of the trough materials. These results suggest that this organism is highly predisposed to adherence regardless of the presence of a pre-existing biofilm. Hall-Stoodley and Lappin-Scott (1998) found that *M. fortuitum* formed biofilms within 48 h and that no other organisms were required for colonization to occur, suggesting that mycobacteria are primary colonizing organisms. On the other hand, when *Map* was re-inoculated into these systems following 365 days of biofilm growth, increases in concentrations of the organism were seen on most all of the trough materials (Table 2). Although these data are preliminary, it appears that in addition to early colonization, the organism is readily incorporated into pre-existing biofilms. This is important in the farm environment since the trough is not static; the trough and associated biofilms would be constantly disturbed and inputs from drinking livestock would occur on a regular basis. It is noteworthy, however, that *Map* concentrations did not increase on concrete. This may be due to the higher concentrations of total bacteria and *Map* present on those materials after 365 days (75–95% higher concentrations than on stainless steel and plastic). However, total and *Map* cell concentrations were not significantly higher than on galvanized steel, which showed a nearly 80% increase in *Map* following re-inoculation. Alternatively, the higher pH and/or chemical composition of the concrete may have influenced *Map* recolonization.

Data from this study show that *Map* readily forms biofilms on livestock watering trough construction materials. Although these were laboratory-based incubation studies, these results provide compelling evidence that *Map* can be rapidly established within mixed-community biofilms on trough materials and can be maintained within the population for long periods of time. There were significant differences in the ability of the organism to remain associated with biofilms on trough materials with the greatest loss of the organism from biofilms occurring on stainless steel, followed by plastic, concrete and galvanized steel. These results suggest that trough water systems may serve as reservoirs for *Map* on farm sites. To inhibit spread of this organism and/or reduce exposure of susceptible animals to *Map* on infected farms, best management practices aimed at maintaining biofilm-free trough surfaces should be included in any John's control plan.

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its approval to the exclusion of other products or vendors that may also be suitable.

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